

# The Cellular Na<sup>+</sup> Pump as a Site of Action for Carbon Monoxide and Glutamate: A Mechanism for Long-Term Modulation of Cellular Activity

J. A. Nathanson, C. Scavone,  
C. Scanlon, and M. McKee  
Neuropharmacology Research Laboratory  
and Department of Neurology  
Harvard Medical School  
Massachusetts General Hospital, CNY-6  
Boston, Massachusetts 02114

## Summary

**Carbon monoxide (CO) induces a long-lasting alteration in cerebellar  $\alpha 3$ -Na,K-ATPase independent of [Na<sup>+</sup>] but linked to cGMP synthesis and localized to Purkinje neurons. The action of CO is absent in Purkinje neuron-deficient mice, mimicked by 8-Br-cGMP, and blocked by inhibition of PKG. Glutamate (Glu) and metabotropic agonists mimic the action of CO, an effect that requires PKC and is associated with CO synthesis. These data suggest that CO regulates Na,K-ATPase through cGMP and PKG, and that Glu regulates CO through mGluRs. This system is also modulated by NMDA agonists and nitric oxide, possibly via Glu release, as well as by free radicals. These findings offer a mechanism by which CO, Glu, and free radicals can exert specific effects on synaptic transmission (relevant to long-term changes in cell excitability), as well as more general actions on energy metabolism (relevant to the pathophysiology of excitotoxicity).**

## Introduction

Carbon monoxide (CO) has been proposed to function as a novel regulator in neural tissue (Marks et al., 1991; Schmidt, 1992; Verma et al., 1993; Ewing et al., 1993), yet very little is known about the possible cellular actions of CO or its presumptive anatomical target(s). Because the constitutive CO synthetic enzyme, heme oxygenase-2 (HO-2), is enriched in selected populations of neurons (Verma et al., 1993; Ewing et al., 1993) and because CO itself is readily diffusible, this gas could act to integrate responses (pre-, post-, or transsynaptic) from multiple temporal or spatial inputs. It could also function to communicate, and perhaps initiate responses to, metabolic changes resulting from regional alterations in cellular activity. The first possibility would have relevance to long-term synaptic alterations, including long-term potentiation (LTP) (Stevens and Wang, 1993), the latter to neuronal homeostasis and related pathological processes, such as acute cerebral ischemia, or the impairment of energy metabolism found in several CNS disorders (e.g., Huntington's, Alzheimer's, and Parkinson's Disease) that may contribute to slow excitotoxic neuronal death (Beal et al., 1993; Brines and Robbins, 1992).

Measurements made in retina indicate that a significant fraction (up to 50%) of the energy metabolism of a neuron (glycolysis plus oxidative processes) may be used for Na<sup>+</sup>

and K<sup>+</sup> ion transport by the membrane-associated Na,K-ATPase (Ames, 1991). Furthermore, recent experiments carried out in kidney and choroid plexus indicate that the intrinsic activity of this enzyme can be regulated by cGMP produced either through activation of atrial natriuretic peptide (ANP) receptor-associated guanylyl cyclase (GC) (Steardo and Nathanson, 1987; Snyder et al., 1992; Scavone et al., 1995) or by stimulation of soluble GC (sGC) by nitric oxide (NO) (McKee et al., 1994). Because CO can activate sGC in a fashion similar to that of NO and has been shown to elevate cGMP levels in peripheral smooth muscle and platelets (Vedernikov et al., 1989; Furchgott and Jothianandan, 1991; Brune and Ullrich, 1987; Ramos et al., 1989), we investigated the possibility that CO might regulate the activity of Na,K-ATPase in brain, and that this enzyme could constitute a potentially important biochemical target for endogenously produced CO as well as other factors activating cGMP production in the CNS. We also wished to determine the mechanism by which such regulation by CO might occur and, finally, whether the endogenous synthesis of CO itself might be subject to regulation by neuronal or hormonal input.

## Results and Discussion

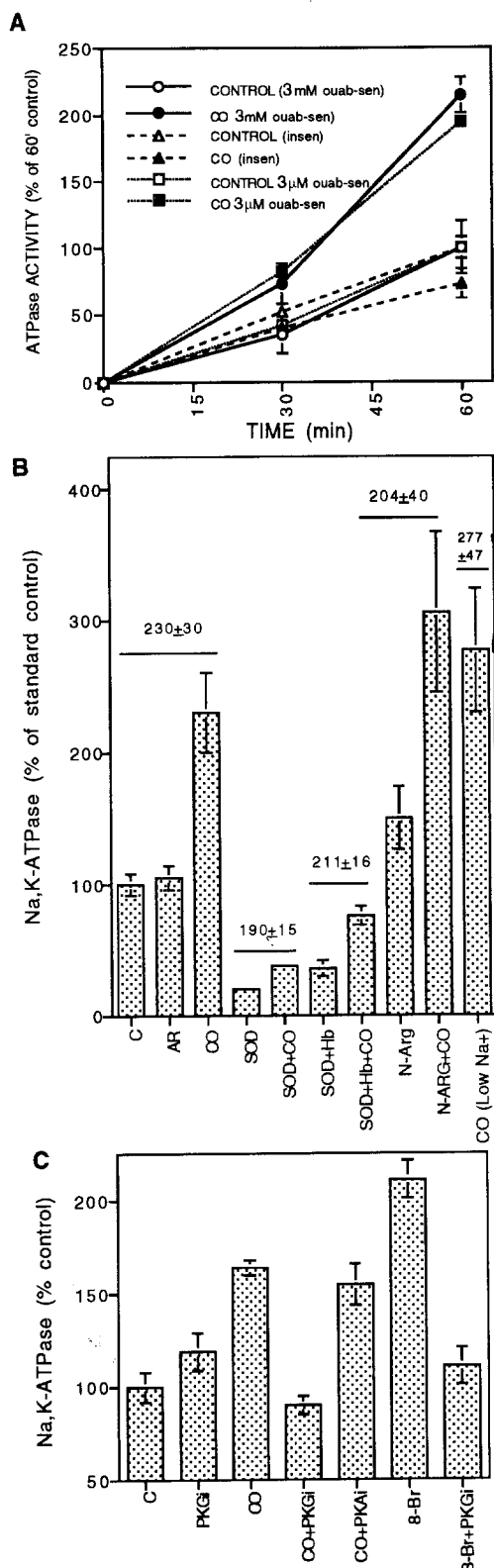
### CO Induces a Persistent Activation of Na,K-ATPase

Experiments were carried out initially in rat cerebellum because of its known enrichment in HO-2 (Verma et al., 1993). Various studies used either tissue slices (incubated in physiological buffer and later permeabilized for measurement of Na,K-ATPase and cGMP), broken cell preparations (for enzyme measurements), or immunocytochemistry.

Following a 15 min incubation of cerebellar slices with CO and then removal of the gas (by washing), Na,K-ATPase activity underwent a substantial (190%–280%) and persistent (>60 min) stimulation compared with that in untreated control slices or slices treated with argon gas (Figures 1A and 1B). Activation by CO was specific for ouabain-sensitive Na,K-ATPase; ouabain-insensitive activity (Mg-ATPase) was not affected (Figure 1A). Furthermore, as determined from ouabain dose–response curves and the known differential affinity of various Na,K-ATPase isoforms for ouabain, >85% of basal and CO-stimulated Na,K-ATPase was calculated to consist of activity from  $\alpha 3$  and/or  $\alpha 2$  isoforms, both of which have a high affinity for ouabain (Sweadner, 1985; Shyjan et al., 1990). Figure 1A shows, for example, that CO activation could be inhibited by 3  $\mu$ M ouabain, a concentration of the glycoside insufficient to demonstrate  $\alpha 1$  isoform activity. Because control Na,K-ATPase activity had been optimized by using saturating concentrations of Na<sup>+</sup>, K<sup>+</sup>, and Mg-ATP, the CO-induced changes observed represented an increase in enzyme maximum velocity.

### CO-Induced Modulation Is Not Due to Alteration of Intracellular [Na<sup>+</sup>]

Prior studies (Thompson and Prince, 1986) have shown



**Figure 1. Regulation of Ouabain-Sensitive Na,K-ATPase by CO**  
(A) Persistent activation of ouabain-sensitive Na,K-ATPase after brief exposure of rat cerebellar slices to CO (100 μM) (closed circles) compared with control (open circles). Ouabain-insensitive activity (Mg-ATPase; open and closed triangles) measured in presence of 3 μM ouabain is unaffected by CO. CO stimulates that portion of Na,K-

that acute changes in neuronal firing can cause acute changes in Na,K-ATPase activity as a result of depolarization-induced increases in [Na<sup>+</sup>] that kinetically activate Na,K-ATPase. However, in the present experiments, Na,K-ATPase was assayed under controlled ionic conditions and only after permeabilization, so that any transient increases in intracellular [Na<sup>+</sup>] that may have occurred secondary to neuronal depolarization, during the prior CO incubation, were eliminated.

Evidence that the slice permeabilization procedure was, in fact, effective in allowing equilibration of charged and normally impermeable molecules was evident from the fact that permeabilized slices allowed intracellular entry of labeled <sup>32</sup>P[ATP] such that quantitative levels of Na,K-ATPase activity measured by this procedure were not significantly different from Na,K-ATPase activity measured directly in broken cell preparations. Also, following permeabilization, cGMP passed freely out of the cell, and levels of intracellular cGMP measured in the incubation wash following freeze-thawing of slices were identical to levels of the nucleotide measured following denaturation of slices by heating for 5 min at 90°C in 75 mM Na acetate, which releases essentially all intracellular cGMP.

Additional evidence that the action of CO on Na,K-ATPase was not due to a kinetic alteration related to intracellular [Na<sup>+</sup>] was obtained from experiments in which slices were exposed to CO (prior to permeabilization) under conditions of low (20 mM) extracellular [Na<sup>+</sup>] that minimized intracellular Na<sup>+</sup> entry. Whereas subsequent basal Na,K-ATPase was somewhat decreased after this exposure, CO still caused a 277% stimulation of basal activity (Figure 1B). This result is consistent with that for ANP-stimulated alterations of Na,K-ATPase in nonexcitable cells in kidney, in which blockade of amiloride-sensitive cation channels fails to prevent ANP-induced modulation of Na,K-ATPase (Scavone et al., 1995).

#### CO Action on Cerebellar Na,K-ATPase Is Independent of the NO System

Because cerebellum is highly enriched in NO synthase (NOS) (for reviews, see Garthwaite, 1991; Snyder and Bredt, 1991), as well as HO-2, and because NO has been shown to regulate Na,K-ATPase in renal medulla (McKee

ATPase activity subject to inhibition by a low concentration (3 μM) of ouabain (squares), indicating an action on the α3 and/or α2 isoforms. (B) Expressed as percentage of basal activity (horizontal lines above bars), stimulation by CO (100 μM) was not affected by SOD (100 U) nor by inhibitors of NO (20 μM Hb; 300 μM N-Arg). Stimulation also occurred in the presence of low extracellular [Na<sup>+</sup>]. Ar, argon. (C) CO stimulation of Na,K-ATPase was blocked by 2 μM KT5823 (PKG), a selective inhibitor of PKG, but not by 0.5 μM KT5720 (PKAi), a selective inhibitor of PKA. Effects of CO were mimicked by 8-Br-cGMP (8-Br; 1 mM), and stimulation of 8-Br was blocked by KT5823. At these concentrations, the two KT compounds had >8-fold selectivity for inhibiting the respective kinases.

Values shown in (A–C) are the mean ± SEM of quintuplicate samples from representative experiments replicated on average 2–3 times. There was a significant increase due to CO in seven experiments under various conditions. (Overall mean stimulation, 259% ± 21%; range, 164%–337%; ANOVA, *p* < .01 in 6 and *p* < .05 in 1).

et al., 1994), it was important to determine whether modulation of Na,K-ATPase by CO requires involvement of NO (stimulated, e.g., by CO-induced depolarization of NO-containing granule cells). Addition of the potent competitive NOS inhibitor N<sup>ω</sup>-nitro-L-arginine (N-Arg; 300 μM), however, did not affect the ability of CO to activate Na,K-ATPase (Figure 1B). Direct assay of NOS (Dawson et al., 1991) confirmed that NO production was nearly completely inhibited by N-Arg under these same conditions.

Additional evidence supporting a role for CO independent of NO involved the known ability of reduced hemoglobin (Hb) to bind at a higher affinity to NO than CO (Marks et al., 1991). Although endogenous levels of NO in cerebellum are unknown, we estimated that, based upon our measurements of NOS activity in cerebellar soluble fractions, [NO] is <10 μM. More specifically, an observed specific activity for NOS of 75–150 pmol/min/mg (a value similar to that reported by others) translates into an average tissue [NO] of 2.2–4.5 μM if one assumes no NO breakdown during incubation. Given this assumption and assuming that free radical concentration does not exceed that of Hb, it was then possible to utilize a Hb concentration of 20 μM that would block the action of NO released endogenously from slices but be too small to sequester more than 20% of exogenous CO, added at 100 μM. Under these conditions (and in the presence of superoxide dismutase [SOD] to prevent inactivation of Hb or NO by O<sub>2</sub><sup>•-</sup>), Hb failed to block the ability of CO to stimulate Na,K-ATPase activity, relative to control (Figure 1B).

Still further evidence of the independence of the action of CO from NO was shown by the marked inhibitory response of Na,K-ATPase to exogenous SOD itself. Because O<sub>2</sub><sup>•-</sup> normally shortens the half-life of NO, SOD would be expected to potentiate effects mediated through NO release. However, SOD's reproducible inhibition of basal activity and lack of effect on CO stimulation suggested that NO was not involved in the ability of CO to activate Na,K-ATPase.

Although any one of the above experiments may have weaknesses, taken together they strongly support an action of CO on Na,K-ATPase that is independent of NO.

#### **The Action of CO Is Independent of Free Radical Regulation of Na,K-ATPase**

Following NMDA receptor stimulation, cerebellar neurons can, in addition to the production of NO, also release O<sub>2</sub><sup>•-</sup> (Lafon-Cazal et al., 1993). In other tissues, O<sub>2</sub><sup>•-</sup> and O<sub>2</sub><sup>•-</sup>-derived radicals, such as peroxynitrite (or S-nitrosothiols) and hydroxyl, are capable of stimulating cGMP production (Mittal and Murad, 1977). Although CO is not itself readily converted to a free radical form, it was important to determine whether the actions of CO on Na,K-ATPase might indirectly involve O<sub>2</sub><sup>•-</sup> production. Figure 1B shows that in the presence of SOD, which rapidly degrades O<sub>2</sub><sup>•-</sup>, CO was still able to stimulate Na,K-ATPase activity (190%–211%) over that seen with SOD alone. As determined from other experiments (data not shown), this concentration of SOD was sufficient to block the cGMP-generating effects of O<sub>2</sub><sup>•-</sup>, mediated through formation of peroxynitrite (or S-nitrosothiols). SOD alone substantially decreased basal

Na,K-ATPase activity, as did inhibitors of the hydroxyl radical. These observations, as well as the fact that xanthine/xanthine oxidase activated the system (data not shown), suggests that free radicals may, independently of CO, affect Na,K-ATPase activity, either directly or indirectly.

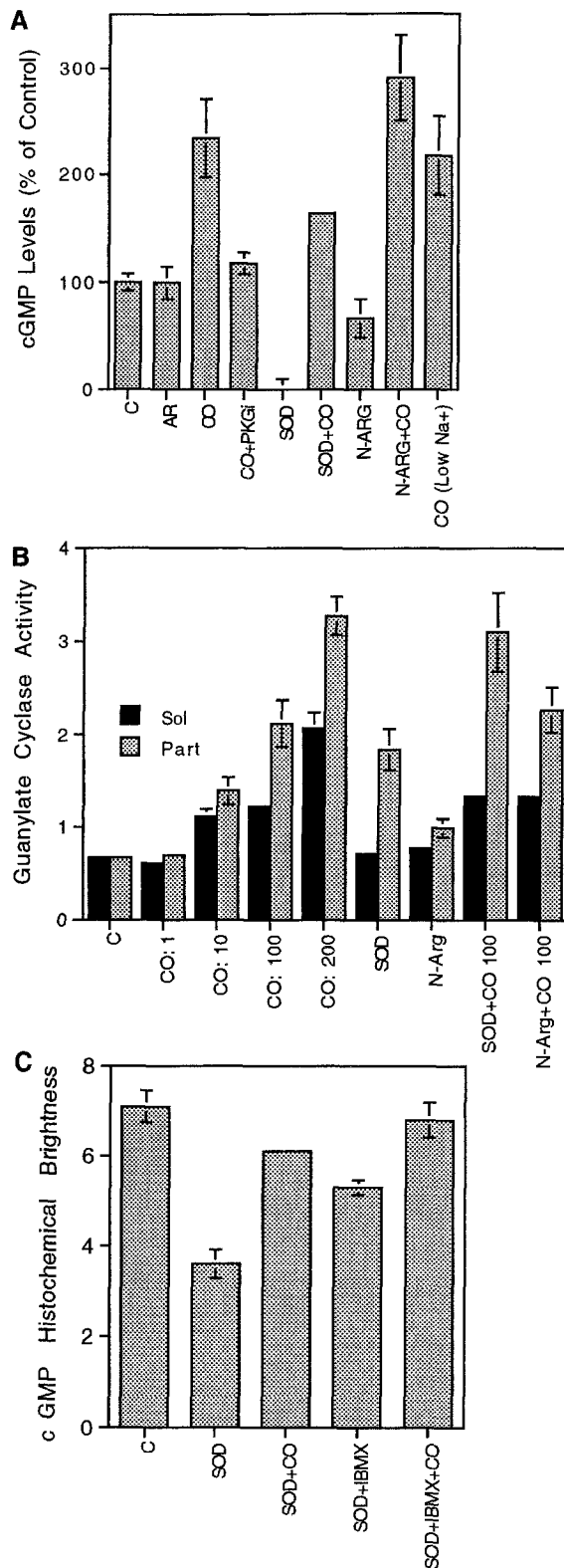
#### **Alterations in cGMP Are Correlated with CO Regulation of Na,K-ATPase: CO Directly Activates Brain GC**

Although independent of NO and O<sub>2</sub><sup>•-</sup>, the activation of Na,K-ATPase by CO was consistently associated with an increase in cGMP, as determined from dual measurements of cGMP and Na,K-ATPase activity in CO-exposed cerebellar slices. Moreover, following a variety of treatments—with CO, SOD, N-Arg, and other agents—alterations in Na,K-ATPase involving both increases and decreases in activity (Figure 1B) were mirrored by corresponding changes in cGMP content (Figure 2A).

Evidence for a direct linkage between CO and cGMP was obtained from assay of GC in cerebellar membrane and soluble fractions. CO caused a dose-dependent activation of sGC activity at CO concentrations similar to those known to relax aortic smooth muscle and inhibit platelet aggregation (Vedernikov et al., 1989; Furchgott and Jothanandan, 1991; Brune and Ullrich, 1987; Ramos et al., 1989; Figure 2B). Furthermore, just as CO was effective in activating Na,K-ATPase in the presence of either N-Arg or SOD, CO was also effective in stimulating sGC activity in the presence of either compound (Figure 2B). Modulation of GC was also observed in cerebellar membrane fractions. This latter result is unusual, as membrane-bound GC is not usually thought to be subject to activation by other diffusible agents such as NO. Therefore, this result should be treated with caution. Nonetheless, these experiments represent the first clear demonstration of CO-activated GC in brain and support an association among CO, cGMP, and Na,K-ATPase.

#### **Possible Involvement of Protein Kinase G**

In kidney, Na,K-ATPase activity modulated by NO and cGMP is blocked by selective inhibition of cGMP-dependent protein kinase (PKG) but not by inhibition of cAMP-dependent protein kinase (PKA) (McKee et al., 1994). This observation is consistent with prior data showing that the PKA substrates DARPP-32 and Inhibitor-1, whose phosphorylation is associated with the cAMP modulation of renal Na,K-ATPase by dopamine, are also good substrates for PKG (Snyder et al., 1992). Supporting the possibility that cGMP and PKG might also modulate effects of CO on altering Na,K-ATPase activity in cerebellum was our observation that KT5823, a selective inhibitor of PKG, but not KT5720, a selective inhibitor of PKA, completely blocked the ability of CO to activate Na,K-ATPase in cerebellar slices (see Figure 1C). (At the concentrations used, the two KT compounds had >8-fold selectivity for inhibiting the respective kinases.) The cGMP analog, 8-Br-cGMP, mimicked the action of CO, causing a similar (210%) stimulation of cerebellar Na,K-ATPase. This activation, too, was blocked by inhibition of PKG (see Figure 1C). When the activity of Na,K-ATPase was compared, under a vari-



**Figure 2. Regulation of cGMP Synthesis of CO**  
CO regulation of Na,K-ATPase is accompanied by a corresponding change in cGMP in brain slices (A) and GC activity in broken cell preparations (B), as well as by a change in cGMP immunofluorescence in PN layer of histological sections prepared from slices (C). (A) Data are from RIA measurements of cGMP present in washes

ety of conditions in various experiments, with simultaneous measurements of cGMP in slices, there was a significant correlation between the amplitude and direction of these two measures ( $r = .87$ ;  $p < .001$ ).

#### CO-Mediated Changes in cGMP Are Localized to Cerebellar Purkinje Cells

Given the potential biochemical link between CO, cGMP, PKG, and Na,K-ATPase, it was of interest to determine whether, in vivo, there is an anatomical association among the components of this putative regulatory system. HO-2 message is known to be enriched in cerebellar granule and Purkinje neurons (PNs) (Verma et al., 1993; Ewing et al., 1993), whereas PNs but not other cerebellar cells also contain high levels of PKG (Schlichter et al., 1980; Nestler and Greengard, 1984). Localization of sGC (Ariano et al., 1982) and cGMP is less clear, as various reports have suggested either glial or neuronal enrichment (Garthwaite, 1991). In the current studies, frozen sections prepared from control slices demonstrated the presence of immunoreactive cGMP in granule cells, blood vessels, and some PN cell bodies (Figure 3A).

Slices stimulated with CO showed a marked increase in cGMP labeling largely restricted to PNs (compare Figures 3B and 3A) and associated with significant enhancement in photometrically quantitated cGMP immunofluorescence measured at the PN/molecular layer interface (see Figure 2C). Stimulation of PN cGMP was better observed in the presence (Figure 3B) than absence (Figure 3C) of SOD, which lowered basal cGMP immunofluorescence, whereas both basal (SOD) and CO-stimulated immunofluorescence were enhanced by the phosphodiesterase inhibitor IBMX. Changes in visual and photometrically determined cGMP immunofluorescence in PNs mirrored previously observed biochemical effects of various agents (e.g., CO, SOD) on cGMP, GC, and Na,K-ATPase (see Figure 1 and Figure 2).

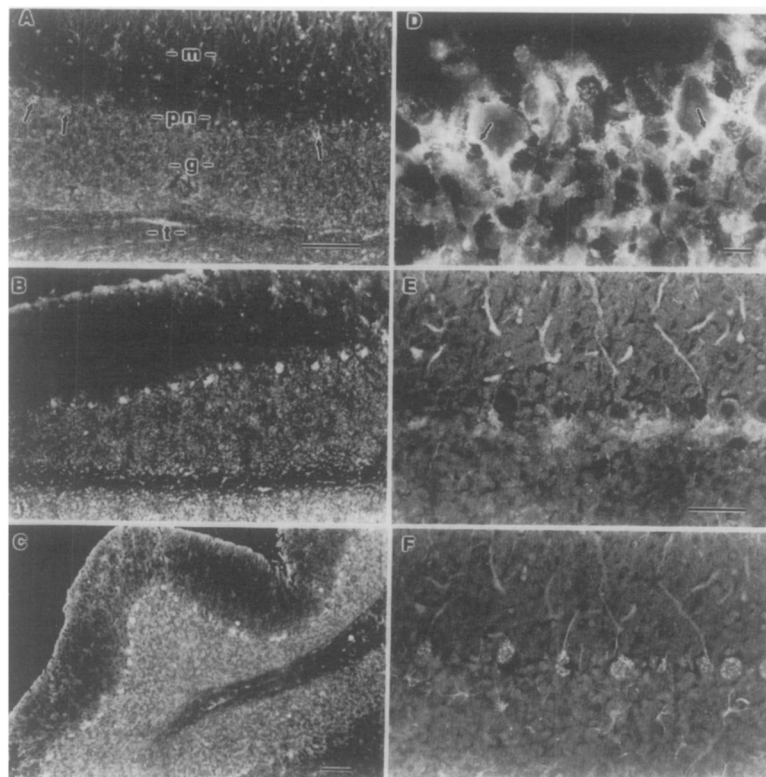
#### CO Modulation May Involve the $\alpha 3$ Isoform of Na,K-ATPase Localized to the PN Layer

Because biochemical studies of ouabain inhibition (see above) indicated that CO-regulated Na,K-ATPase in rat

derived from permeabilized slices (see Figure 1). Values are from typical experiments and represent mean  $\pm$  SEM of triplicate samples, each assayed for cGMP in duplicate ( $p < .01$  for CO stimulation relative to corresponding control).

(B) GC activity present in rat cerebellar supernatant and particulate fractions. Values represent mean  $\pm$  range of duplicate samples each assayed for cGMP in triplicate. CO:1, 1  $\mu$ M CO; CO:10, 10  $\mu$ M; CO:100, 100  $\mu$ M; CO:200, 200  $\mu$ M. Other symbols as in Figure 1.

(C) Cryostat sections (20  $\mu$ m) of control and drug-treated cerebellar slices were immunostained for cGMP and fluorescence quantitated on an Olympus BX-50 microscope with a PM-20 photographic photometer. Values shown (arbitrary units) are mean  $\pm$  SEM of 4–5 measurements per condition. Histochemical experiments were replicated three times. For control vs. SOD, SOD vs. SOD + CO, SOD vs. SOD + IBMX, and SOD + IBMX vs. SOD + IBMX + CO,  $p < .01$ . Drug-induced changes, though significant, were relatively small, probably because most soluble cGMP is removed during histochemical processing, and that seen by immunofluorescence represents primarily bound cGMP.



**Figure 3.** Immunohistochemical Localization of CO-Stimulated Increases in cGMP and the  $\alpha 3$  Isoform of Na,K-ATPase in Rat Cerebellum  
(A) Control prepared from cerebellar slice incubated in SOD alone (to decrease basal cGMP) showing (top to bottom) molecular layer (m), PN (pn) layer, granule cell layer (g), and white matter tract (t). (Apparent staining of basket and stellate cells is largely nonspecific.)  
(B) Following 15 min exposure to 100  $\mu$ M CO, there is a marked increase in cGMP immunostaining of PN, shown aligned at junction of molecular and granule cell layers.  
(C) Lower power view of section from slice treated with CO in absence of SOD, showing somewhat higher levels of immunostaining in all areas.  
(D) Enrichment of  $\alpha 3$  isoform of Na,K-ATPase in plasma membrane of PN cell bodies (arrows), nearby granule cells, and areas surrounding proximal portions of PN axons.  
(E and F) In sections first reacted with NADPH-d to quench immunofluorescence in NOS-containing cells, and then dual labeled, both  $\alpha 3$ -Na,K-ATPase (shown by rhodamine optics in [E]) and cGMP (shown by fluorescein optics in [F]) are well visualized in PN dendrites. cGMP is also seen in astroglia (overlying granule cells) and in region of PN basket cell synaptic endings.  
Bars, 100  $\mu$ M (A-C); 10  $\mu$ M (D); 50  $\mu$ M (E and F).

cerebellum involves primarily  $\alpha 3$  and/or  $\alpha 2$  isoforms, it was of interest to determine localization of Na,K-ATPase isoforms in incubated slices. Isoform-selective antibodies (McGrail and Sweadner, 1991) revealed that, as suggested by in situ hybridization and high affinity ouabain binding (Brines et al., 1990; Hieber et al., 1991), all three Na,K-ATPase isoforms are to varying degrees present in granule cells; however, PNs contained only  $\alpha 3$  immunofluorescence.  $\alpha 3$  was present in the plasma membrane of PN cell bodies (Figure 3D) and in an area under each cell body near the PN axon hillock and infraganglionic plexus of recurrent PN axons (Figures 3D and 3E), a localization that coincides with areas in cerebellar cortex known to be enriched in PKG (Nestler and Greengard, 1984).

Clearer localization of  $\alpha 3$  and its anatomic relationship to cGMP was obtained by selectively reducing immunofluorescence in cells of the NO system by using the NOS marker NADPH-diaphorase (NADPH-d). The dense deposition of nitroblue tetrazolium reaction product in NOS-containing cells largely quenched fluorescence in those cells and revealed that  $\alpha 3$  (Figure 3E) and cGMP (Figure 3F) both colocalize in proximal PN dendrites (which do not react for NADPH-d). PN cell bodies also contained both  $\alpha 3$  (Figure 3D) and cGMP (Figure 3B), and NADPH-d quenching revealed intense cGMP reactivity in the region of PN basket cell synaptic endings (Figure 3F), as well as in some astroglia and a few axons ascending from the granule cell layer (data not shown). The apparent localization of both  $\alpha 3$  and cGMP in PN cell bodies and proximal (as opposed to distal) PN dendrites was of interest from a functional point of view, given recent observations of

proximal versus distal dendritic differences in PN  $\text{Ca}^{2+}$  mobilization observed during studies of LTD (Shigemoto et al., 1994). It was also intriguing that this localization was similar to that observed for the distribution of PKC following stimulation by (1S,1R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD; see below and Figure 7). Nonetheless, it would be premature to rule out the presence and involvement of  $\alpha 3$  and cGMP elsewhere in cerebellum. It should also be noted that the quenching technique described above enhances the detail and contrast particularly in cells containing low levels of NOS. The method will tend to obscure immunoreactivity in cells enriched in NOS.

Further support for the PN as a possible site of CO- and cGMP-mediated changes in Na,K-ATPase activity was obtained with *nervous* mutant mice (Balb/cGr-nr/nr), whose cerebella, as demonstrated by histological studies (data not shown), were markedly deficient (90%) in PNs (Sidman and Green, 1970). Biochemical studies of slices from mutant nr/nr cerebella compared with slices from phenotypically normal litter mates showed loss of both CO-induced cGMP increases and CO-induced alterations in Na,K-ATPase.

#### Exogenous Versus Endogenous Activation of the CO/cGMP/ATPase System

The observed regulation of Na,K-ATPase activity by exogenous CO could be a reflection of in vivo events or simply an in vitro phenomenon resulting from two interesting but causally unrelated factors: the reported ability of endogenous NO to regulate Na,K-ATPase through stimulation of cGMP formation (McKee et al., 1994) and the capability

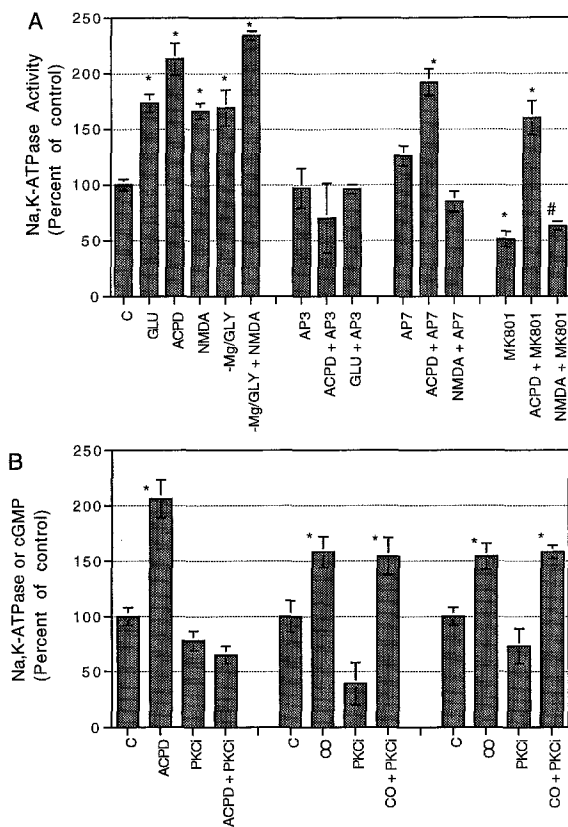


Figure 4. Glutamate Regulates Na,K-ATPase via mGluRs and PKC (A) Effect of Glu agonists and antagonists on ouabain-sensitive Na,K-ATPase activity in slices of rat cerebellum. Data suggest that NMDA activation occurs indirectly through stimulation of mGluRs, possibly via release (see text). Values are mean  $\pm$  SEM of quintuplicate samples. GLU, 300  $\mu$ M Glu; ACPD, 100  $\mu$ M; NMDA, 100  $\mu$ M; -Mg/Gly, magnesium-free buffer with 3  $\mu$ M glycine; AP-3, 100  $\mu$ M; AP-7, 100  $\mu$ M; MK801, 1  $\mu$ M. Asterisk,  $p < .01$ , significantly different than control; double asterisks, not significantly different from MK801. (B) Selective inhibition of ACPD- but not CO-activation of Na,K-ATPase by KT252b, a relatively selective inhibitor of PKC. Note that cGMP levels (four bars at right) mimic pattern of changes in Na,K-ATPase (four center bars), supporting downstream action of CO on GC. ACPD and CO, 100  $\mu$ M; KT252b, 40 nM. Values for Na,K-ATPase are mean  $\pm$  SEM of 5 samples, whereas cGMP values are means  $\pm$  range of replicate samples, each assayed in duplicate.  $p < .01$ , relative to control or PKCi alone.

of exogenous CO to mimic the cGMP-promoting action of endogenous NO by binding tightly to the heme regulatory site of sGC. However, there are several observations that support an endogenous and independent role for CO. First, CO is synthesized in nerve tissue (Maines, 1988); second, this synthesis occurs in discrete regions of the brain not identical to those containing NOS; and third, sites of cGMP synthesis correlate better with the distribution of HO-2 immunoreactivity than with NOS immunoreactivity (Verma et al., 1993).

On an absolute basis, whole brain (along with spleen and testis) contains the highest organ levels of HO-2 activity in rat (Maines, 1988). Furthermore, it can be calculated that the endogenous specific activity in brain (85  $\mu$ mol/l/min in the high speed tissue pellet) is sufficient, given adequate

substrate, to produce CO at levels at least as great as those used in vitro to stimulate GC and alter Na,K-ATPase activity in cerebellar slices (see Figure 1 and Figure 2). Given that these calculations are based on whole brain and that the specific activity of HO-2-enriched regions (e.g., cerebellar cortex) is actually greater, local synaptic concentrations of CO are likely to be considerably higher. Thus, even though diffusion and endogenous binding may diminish levels, concentrations of CO produced in vivo are almost certainly adequate to mediate the changes in Na,K-ATPase described.

The fact that HO-2 is distributed nonuniformly among functionally distinct neuronal subtypes lends further support to an endogenous regulatory role for CO, as do our observations that stimulation of cerebellar slices with CO (Figure 3) leads not to a generalized increase of cGMP in all GC-containing cells, but rather to preferential stimulation of cGMP in PNs. There is also evidence for tonic, basal production of CO, independent of NO, as shown by the report (Verma et al., 1993) that addition of the relatively selective HO-2 inhibitor zinc protoporphyrin-9 (ZnPP-9) to neurons decreases cGMP levels, whereas the NOS inhibitor N-Arg fails to alter cGMP. Similarly, in the present studies, the stimulatory effect of CO on Na,K-ATPase could not be blocked by N-Arg (see Figure 1), even at concentrations able to inhibit >95% of endogenous NOS activity (data not shown). However, the most compelling evidence for an endogenous role of CO in regulating Na,K-ATPase are observations (see below) that the afferent PN transmitter glutamate (Glu), acting at metabotropic Glu receptors (mGluRs), stimulates cerebellar Na,K-ATPase through regulation of HO-2 activity.

#### Endogenous Regulation of cGMP and Na,K-ATPase by Glu and mGluRs

Localization of CO-induced changes in cGMP to PNs, together with enrichment of HO-2 and  $\alpha 3$  Na,K-ATPase in these cells, raised the possibility that endogenous afferents to PNs, acting through PN HO-2, might normally play a role in regulating long-term changes in PN Na,K-ATPase. Glu constitutes the major excitatory input to PNs and acts directly at AMPA and mGluRs (especially mGluR1) located on PN dendrites and cell bodies. Glu also acts indirectly on PNs through excitation of granule cells, which contain AMPA, N-methyl-D-aspartate (NMDA), and possibly some mGluR4s (Blackstone et al., 1989; Garthwaite, 1991; Tanabe et al., 1992). Of these receptor types, mGluRs (particularly mGluR1) show a relatively selective localization to PNs compared with other cerebellar neurons, as shown directly (Tanabe et al., 1992) and as evidenced by a marked and preferential loss of mGluRs in PN-deficient *nervous* mutant mice and in olivopontocerebellar atrophy, characterized by degeneration of cerebellar cortical cells, especially PNs (Makowiec et al., 1990).

Exposure of slices to Glu caused a significant increase in Na,K-ATPase activity (173%  $\pm$  8% of control; mean  $\pm$  SEM,  $n = 5$  experiments; Figure 4A) selective for ouabain-sensitive activity, and both linear and stable (in the absence of drug) for at least 60 min. The effect of Glu was mimicked by the mGluR agonist ACPD which more than

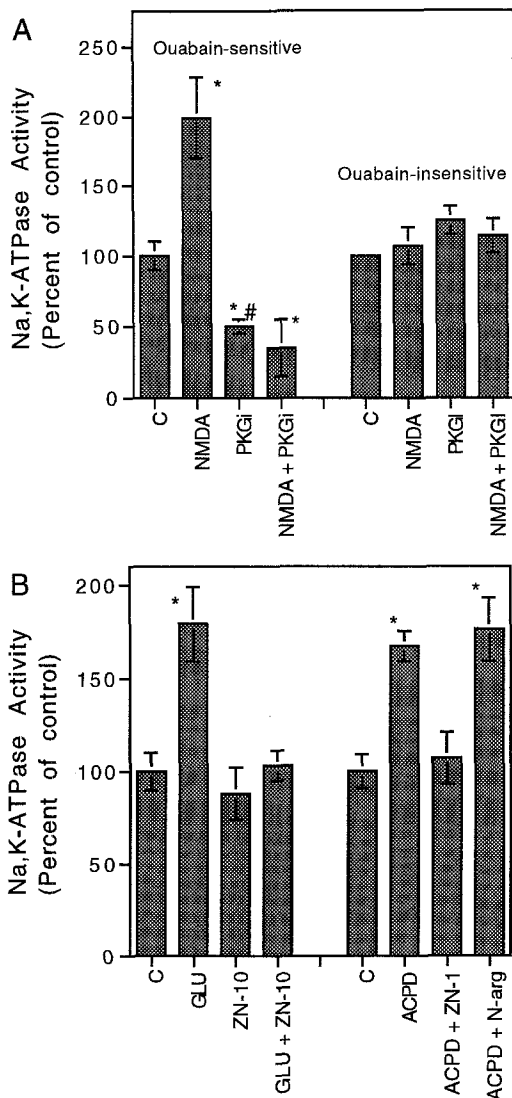


Figure 5. Effects of Glutamate on Na,K-ATPase Require PKG and HO-2

(A) Ouabain-sensitive but not -insensitive Na,K-ATPase is activated by NMDA (100  $\mu$ M), and stimulation is blocked by 2  $\mu$ M KT5823 (PKG), a relatively selective inhibitor of PKG. Values are mean  $\pm$  SEM of five samples. Asterisk,  $p < .01$  different from control.

(B) Inhibition of Glu and ACPD activation of Na,K-ATPase by 10  $\mu$ M or 1  $\mu$ M ZNPP-9 (ZN-10 or ZN-1), a relatively selective inhibitor of HO-2. Together with Glu stimulation of CO production (see below), these data suggest that Glu agonists acting via mGluRs may regulate CO synthesis in CNS. Note that the NOS inhibitor, N-Arg, does not block ACPD (100  $\mu$ M) stimulation, consistent with scheme shown in Figure 6. Values are mean  $\pm$  SEM for five replicates. GLU, 300  $\mu$ M. Asterisk,  $p < .01$ . Pound sign, in some experiments KT823 did not affect basal activity.

doubled basal activity at concentrations known to be selective for mGluRs. The action of ACPD on Na,K-ATPase was blocked by the relatively mGluR-selective antagonist AP-3, whereas the relatively selective NMDA antagonist AP-7 and the ionotropic channel blocker MK801 had no effect on ACPD-regulated Na,K-ATPase.

On the other hand, enzyme activity was also stimulated

following exposure to NMDA or after incubation of slices under conditions (added glycine and  $Mg^{2+}$ -free buffer) that enhance NMDA receptor/channel activation (Figure 4). NMDA stimulation was blocked by concentrations of AP-7 or MK801 that were unable to block ACPD (Figure 4A). These results indicate that NMDA receptor activation can also, either directly or indirectly, lead to prolonged modulation of Na,K-ATPase. An indirect effect of NMDA, possibly acting through Glu release and stimulation of mGluRs (see references below), is consistent with the observation that addition of Glu itself (which affects both NMDA and mGluRs) activated Na,K-ATPase, and this activation could be completely blocked by using the mGluR antagonist AP-3, alone (Figure 4A). The effect of NMDA could also be blocked by AP-3 (data not shown), and similar to the action of CO (see Figure 1C), the stimulatory effect of NMDA on Na,K-ATPase was blocked by inhibition of PKG (Figure 5A).

Thus, though antagonism of mGluRs could block the effects of NMDA receptor activation on Na,K-ATPase, the converse was not true. Therefore, NMDA appears to act through, or require the participation of, mGluRs to bring about the prolonged alteration in Na,K-ATPase activity observed. The action of ACPD, however, acting at mGluRs may not require participation of NMDA receptors.

As was observed for CO, cerebellar slices from mutant *nervous* mice deficient in PNs showed a loss of ACPD regulation of Na,K-ATPase (data not shown), suggesting that the PN may serve as one possible site for the observed metabotropic regulation of Na,K-ATPase. Such a localization is consistent with the enrichment in PNs of PKC-linked mGluRs relative to NMDA receptors, with the converse being true in afferent granule cells and presynaptic Glu inputs.

Activation of afferent or presynaptic NMDA receptors can cause enhanced neurotransmitter release through a mechanism involving calcium-stimulated production of NO (Montague et al., 1994; Meffert et al., 1994). Specifically, NMDA can initiate NO-dependent release of Glu from cultured granule cells (Oh and McCaslin, 1994). Together with the above pharmacological data, these latter findings suggest that the observed activation of Na,K-ATPase by NMDA may result, at least in part, from stimulation of granule cells and/or release from terminals of Glu, which then acts (directly or indirectly) on mGluRs located postsynaptically on PNs (Figure 6).

#### Metabotropic Regulation of Na,K-ATPase May Involve PKC and HO-2-Mediated CO Formation

Stimulation of PN mGluR1s is associated with activation of PKC and  $IP_3$  production, rather than cGMP synthesis (see Tanabe et al., 1992 for references). Therefore, it was surprising to observe that, concomitant with activation of Na,K-ATPase, ACPD elevated cGMP in cerebellar slices ( $194\% \pm 33\%$ ; mean  $\pm$  SEM) nearly as much as was observed with CO (see Figure 2). Moreover, ACPD, like CO, increased cGMP immunofluorescence in PNs. These results, together with those above indicating that CO can, like ACPD, stimulate both cGMP and Na,K-ATPase, suggest that metabotropic regulation of Na,K-ATPase might result from an effect of mGluRs on CO synthesis or action.

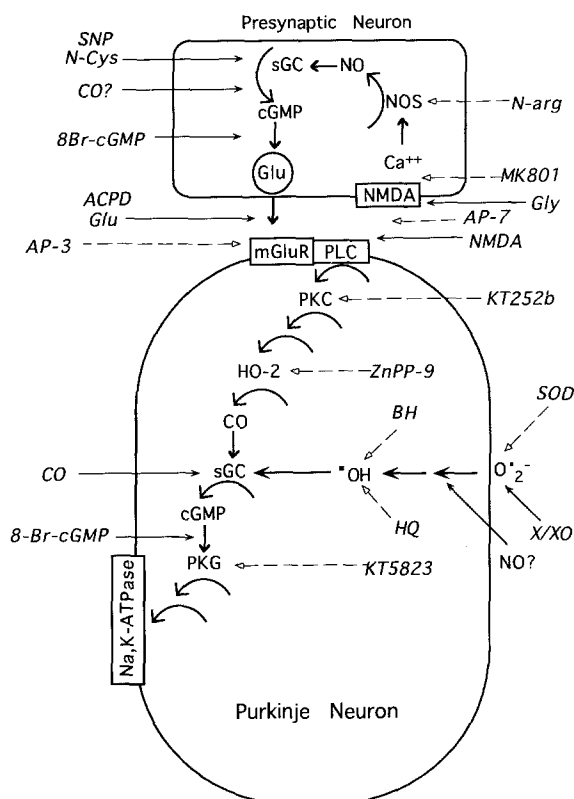


Figure 6. Diagram Describing Postulated Regulated Pathway Involving CO

Glu, acting at mGluRs and PKC, stimulates CO synthesis by HO-2. CO then activates Na,K-ATPase through stimulation of GC and activation of PKG. PKC activation of HO-2 and PKG activation of Na,K-ATPase may require more than a single step. In this scheme, NMDA and NO act indirectly and presynaptically, primarily through regulation of Glu release. Oxygen free radicals may also regulate this system by stimulating sGC independently of the Glu/CO path. This scheme does not rule out additional presynaptic actions of CO or postsynaptic actions of NO and is consistent with the observed effects of pharmacological agents utilized in the current studies. Small closed arrowheads indicate site of action of various agonists, and open broken arrows indicate site of action of antagonists.

SNP, Na<sup>+</sup> nitroprusside; N-Cys, nitrocysteine; Gly, glycine; PLC, phospholipase C; BH, butylated hydroxyanisole; HQ, hydroquinone.

Such a mechanism is consistent with the report that the HO-2 inhibitor ZnPP-9 blocks the electrophysiological effects of ACPD in brainstem (Bashier and Henley, 1993) and the observation that mGluRs and CO influence the establishment of hippocampal LTP and/or PN LTD (Linden et al., 1993; O'Conner et al., 1994; Stevens and Wang, 1993). Others have shown that LTP and LTD may also be associated with PKC (Shigemoto et al., 1994). Because mGluR1s can activate PKC and because these receptors and HO-2 are both present in PNs, it was of interest to investigate the possibility that the actions of ACPD on Na,K-ATPase might involve activation of PKC.

#### Anatomical Distribution of PKC Immunoreactivity in Cerebellum and Changes Following Treatment with Glu Agonists and SOD: Intracellular Redistribution and Marked Cell-to-Cell Variation among Individual PNs

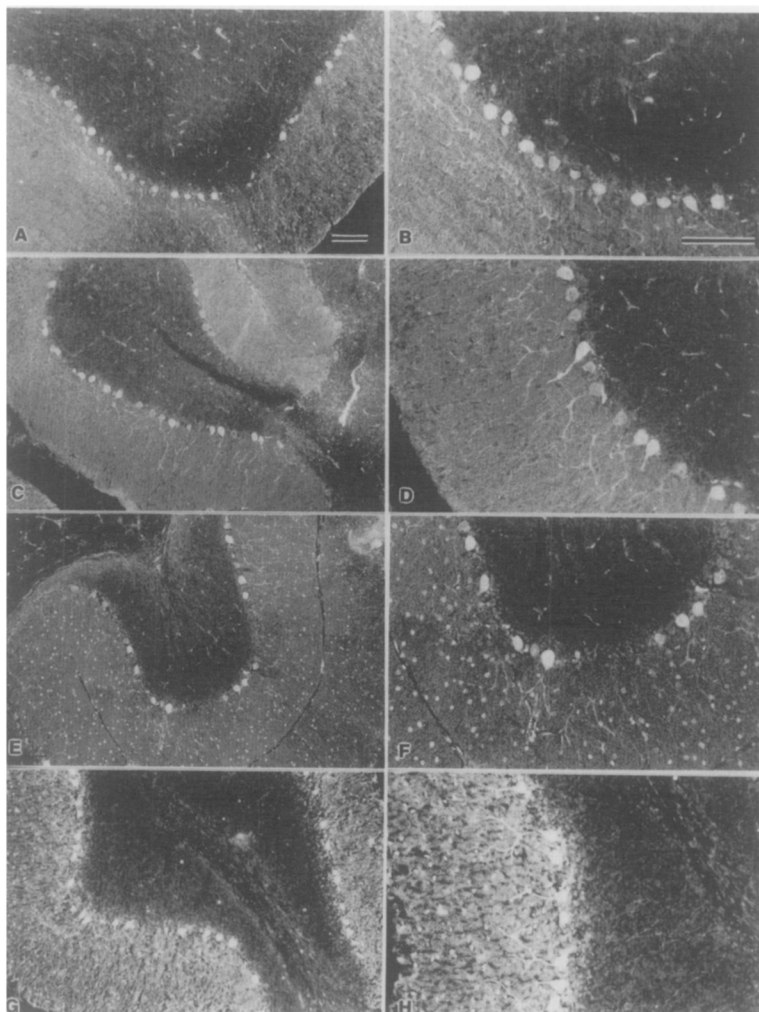
Immunostaining for the neuronally enriched  $\gamma$  isoform of PKC was carried out in SOD-treated cerebellar slices. Under these conditions, controls revealed a striking enrichment of PKC in PN cell bodies (Figure 7A and 7B), with only modest staining of proximal PN dendrites and considerable activity distributed homogeneously throughout the molecular layer. Granule cell and fiber layers were largely negative, except for nonspecifically staining blood vessels.

Following treatment with ACPD, although overall intensity of staining remained relatively unchanged, there was a redistribution of immunofluorescence, with a substantial increase in labeling of PN proximal dendrites (Figures 7C and 7D). Though this change was consistent with the known ability of PKC to undergo subcellular redistribution following activation, of particular interest was the fact that PNs demonstrated marked individual variability in the distribution of PKC immunoreactivity. Following ACPD, some cells showed bright homogenous cytoplasmic staining, others predominantly membrane staining, and in still others, staining was absent from the cell body but prominent in proximal dendrites. Such variability indicates an inherent heterogeneity among PNs in the state or responsiveness of individual neurons and/or PKC to ACPD activation and is consistent (as one would expect from a coding mechanism) with a role for PKC and PNs in cerebellar information processing and storage. These immunohistochemical observations also support the hypothesis that ACPD modulation of Na,K-ATPase is associated with stimulation or modulation of PN PKC.

NMDA caused an increase in PN dendritic PKC immunoreactivity similar to, but somewhat less marked than, that seen with ACPD (Figures 7E and 7F). NMDA exposure also caused the same cell-to-cell variability as ACPD. However, quite distinct from ACPD action was prominent staining of molecular layer interneurons (basket and possibly stellate cells). Thus, NMDA stimulation can affect cell types presynaptic to PNs, a finding consistent with the observed pharmacological characteristics of NMDA stimulation of Na,K-ATPase (above), as well as studies (Oh and McCaslin, 1994) demonstrating NMDA-stimulated release of Glu from granule cells, which are known to synapse not only on PNs, but also on basket and stellate cells.

Relevant to the observed effects of CO on Na,K-ATPase was the finding that omission of SOD resulted in elevation, not only of basal cGMP (see Figure 2 and Figure 3), but also in the intensity of PKC immunoreactivity seen in all areas of the molecular layer, including PN cell bodies, dendrites, and basket and stellate cells (Figures 7G and 7H). Without SOD, there was also loss of the individual variability seen among PNs. This action of SOD, which is opposite to that manifested by SOD in NO-regulated systems, supports a role for free radicals and/or CO and suggests that in SOD-deficient slices, endogenous  $O_2^{\cdot -}$





**Figure 7. Immunocytochemical Localization of PKC  $\gamma$  in Drug-Treated Cerebellar Slices**

Shown are low (left) and high (right) power views of slices treated for 15 min with vehicle (A and B), 100  $\mu$ M ACPD (C and D), 100  $\mu$ M NMDA (E and F), and by omission of SOD (G and H) present in other slices at 100 U/ml. Note enhanced labeling of PN dendrites with ACPD and NMDA, as well as marked cell-to-cell variability in PKC labeling. Lack of free radical scavenger results in marked increase in immunolabeling throughout molecular layer. Bar, 100  $\mu$ M.

formation can occur, a possibility consistent with data directly demonstrating Glu-stimulated formation of  $\text{OH}^\cdot$  and  $\text{O}_2^{\cdot-}$  in cultured granule cells (Lafon-Cazal et al., 1993). Because of the marked effects of SOD on both cGMP and PKC, as well as Na,K-ATPase in vitro, this result also raises the possibility of a potential physiological or pathological role for free radicals in regulating neuronal Na,K-ATPase in vivo.

#### **Biochemical Experiments: Effects of PKC Inhibition and HO-2 Inhibition on ACPD Activation of Na,K-ATPase**

Addition of the relatively selective PKC inhibitor KT252b to cerebellar slices completely blocked ACPD stimulation of Na,K-ATPase (see Figure 4B). This is consistent with the above hypothesis, indicating involvement of PKC in mediating the actions of Glu and mGluR activation on long-term regulation of Na,K-ATPase.

Whereas KT252b completely blocked ACPD action, this inhibitor had no effect on CO stimulation of Na,K-ATPase or cGMP (Figure 4B), results that not only ruled out non-

specific effects being involved in the action of KT252b in blocking ACPD (above) but suggested that CO regulation of Na,K-ATPase most likely occurs distal to (i.e., after) the action of ACPD on PKC (see Figure 6). Although the action of CO on Na,K-ATPase was not blocked by PKC inhibition, it was blocked by inhibition of PKG (see Figure 1); therefore, the CO pathway described here appears distinct from the reported inhibitory effects of PKC on Na,K-ATPase in kidney (Katz et al., 1993). Nonetheless, it is possible that such inhibitory effects of PKC could also exist in cerebellum, given that addition of phorbol ester (100 ng/ml) to cerebellar slices resulted in a  $55\% \pm 18\%$  inhibition of basal Na,K-ATPase (data not shown). This latter result needs to be studied in more detail but, if real, these data raise the possibility of bidirectional regulation of neuronal Na,K-ATPase. Dual regulation by PKC and a cyclic nucleotide kinase has been observed previously with other substrates, such as DARPP-32 (Halpain et al., 1990). Clearly, additional experiments are necessary to elucidate both direct actions of PKC on Na,K-ATPase, as well as indirect effects that may be mediated through activation of HO.

Table 1. Stimulation of cGMP by Glu and ACPD Can Occur in the Presence of Inhibitors of NOS and Oxygen Free Radicals but is Blocked by Inhibition of Heme Oxygenase

Drug ( $\mu$ M)	Increase in cGMP over Control of Inhibitor Alone	
	Percent	Absolute (fmol/mg)
<b>A</b>		
Glu (300)	142 $\pm$ 15	680 $\pm$ 120
Glu + Zn-PP9 (10)	63 $\pm$ 23	250 $\pm$ 100
ACPD (100)	91 $\pm$ 12	650 $\pm$ 90
ACPD + N-Arg (300)	92 $\pm$ 14	640 $\pm$ 90
<b>B</b>		
Glu (300)	26 $\pm$ 8	580 $\pm$ 200
Glu + Zn-PP9 (10)	0 $\pm$ 5	0 $\pm$ 150
ACPD (100)	57 $\pm$ 8	1300 $\pm$ 190
ACPD + Zn-PP9 (10)	0 $\pm$ 5	0 $\pm$ 250

For the experiment in A, drugs at left were added to standard buffer as shown. In B, buffer for all conditions contained the following additional inhibitors: 100 U/ml SOD, 100 U/ml catalase, 300  $\mu$ M N-arg, and 0.5 mM IBMX. Values shown are mean  $\pm$  range of triplicate samples, each assayed for cGMP in duplicate.

As noted (see Figure 5), the effect of NMDA on Na,K-ATPase could also be blocked by inhibition of PKG. This and the above biochemical results, together with the colocalization of HO-2, PKC, and mGluRs in PNs, raise the possibility that the glutamatergic and CO effects observed might be connected; more specifically, that ACPD (via PKC) might activate Na,K-ATPase (either directly or indirectly) by stimulating CO formation or action (see Figure 6).

To investigate this, the relatively selective HO-2 inhibitor ZnPP-9 was added to slices in the absence or presence of Glu agonists. In four separate experiments, ZnPP-9 consistently blocked the ability of either Glu or ACPD to activate Na,K-ATPase (see Figure 5B). In three experiments, ZnPP-9 used alone had no effect on basal Na,K-ATPase or cGMP levels (causing a small stimulation in the fourth).

To rule out the possibility that the action of ZnPP-9 might be through direct inhibition of GC or NOS, a 10-fold lower concentration (1  $\mu$ M) of the metalloporphyrin was used. This dose of ZnPP-9 blocks the action of CO (Verma et al., 1993) but is much less than that (20  $\mu$ M) necessary to cause even a small inhibition of cGMP or NOS (Haley et al., 1994). ZnPP-9 (1  $\mu$ M) completely blocked Na,K-ATPase stimulation by ACPD (see Figure 5B). In contrast, the NOS inhibitor N-Arg failed to inhibit ACPD (see Figure 5B), even at concentrations (300  $\mu$ M) at which it completely inhibited NOS synthesis (data not shown). Finally, it was noted that ZnPP-9, though effective in completely blocking activation of Na,K-ATPase by Glu, partially, but did not completely, block the Glu-stimulated increase in cGMP (Table 1). This result is consistent with the ability of Glu to activate NMDA receptors in addition to mGluRs; the former, through  $\text{Ca}^{2+}$ -activated NO production, can stimulate sGC at non-PN sites. If ZnPP-9 were acting by direct inhibition of sGC, cGMP elevations should have been totally blocked in slices. These studies with ZnPP-9, therefore, support the possibility that ACPD and Glu may modulate Na,K-ATPase through an action on HO.

Table 1 also demonstrates that the pathway that involves HO-2 and CO for generating cGMP is distinct from that involving NOS and NO. Part A in Table 1 shows that N-Arg, used at a concentration that completely inhibits NO production, had no effect whatsoever on blocking ACPD stimulation of cGMP formation. Conversely, as noted above, ZnPP-9 only partially inhibited cGMP production because Glu could still affect NMDA receptors.

In part B of Table 1, slices were placed in medium containing N-Arg at levels sufficient to block NOS and inhibit formation of cGMP by NO. The media also contained SOD and catalase to prevent GC activation by  $\text{O}_2^{\cdot -}$ . Under such conditions, Glu was still effective in stimulating cGMP. Because the action of Glu via the NMDA NO pathway was inhibited, the degree of stimulation by Glu was reduced but was still statistically significant ( $p < .05$ ). ACPD was relatively more effective in stimulating cGMP production under these conditions. With ZnPP-9, however, effects of both Glu and ACPD could be completely blocked. As mentioned (see Figure 5B), similar concentrations of ZnPP-9 block the ability of ACPD and Glu to activate Na,K-ATPase. These results add support to the possibility that HO-2 and cGMP are involved in the actions of Glu on modulating PN Na,K-ATPase.

#### Biochemical Experiments: Direct Demonstration of Hormonal Regulation of CO Production Using a New CO Capture Assay

To provide further evidence for linking Glu agonists and HO-2, a spectrophotometric method was developed to assay directly the rate of CO production. This assay determined the rate of formation of carboxyhemoglobin (Hb-CO) produced from CO, made in slices that then combined with oxyhemoglobin present in a compartmentalized form in red blood cells (RBCs) suspended in the surrounding medium (see Experimental Procedures). Based upon prior observations, CO production in brain can be attributed almost exclusively to HO (primarily HO-2) (Maines, 1988; Marks et al., 1991), with a small contribution from other sources (Schmidt, 1992); therefore, measurement of CO production yields data on the activity of HO-2. The capture assay showed minimal interference from NO and offered the advantage of directly measuring CO, as compared with some existing methods (Maines, 1988), which measure the secondary conversion of the HO-2 coproduct, biliverdin, to bilirubin. Sequestration of the capture agent in RBCs further prevented any potential interactions of Hb with the enzyme.

Using the Hb-CO method, we detected a total basal CO production rate of  $3.4 \pm 0.6$  nmol/mg/hr in nonenriched rat cerebellum. This activity compares well with the value of 10.6 nmol/mg/hr reported by Maines in HO-2-enriched rat brain microsomal fractions. Cerebellar CO production was completely inhibited by 10  $\mu$ M ZnPP-9, consistent with synthesis by HO-2. Furthermore, in the presence of 500  $\mu$ M Glu, the rate of CO production increased by  $171\% \pm 28\%$  in one experiment and by  $141\% \pm 14\%$  in another experiment. In a third experiment, at 1 mM Glu, CO synthesis was stimulated by  $247\% \pm 40\%$ .

These results provide direct evidence that Glu can acti-

vate CO synthesis under the same conditions in which it alters Na,K-ATPase activity. Thus, it can be concluded that CO activates Na,K-ATPase, Glu mimics this effect, and Glu stimulates the formation of CO. Coupled with the above histochemical and pharmacological data, these results support the possibility that afferent input to PNs can initiate a prolonged alteration of Na,K-ATPase activity through a pathway linking the mGluR/PKC system to cGMP/PKG via stimulation of HO-2 and production of CO (see Figure 6).

#### **Activation of Na,K-ATPase by NO and Free Radicals**

Based on the scheme in Figure 6, several testable predictions can be made. One is that NO agonists, which are known to mimic the action of NMDA presynaptically (Montague et al., 1994; Meffert et al., 1994), should, via stimulation of Glu release onto PNs, also stimulate Na,K-ATPase. Utilizing the same conditions as those used previously, we observed that the NO agonists sodium nitroprusside and nitrocysteine caused substantial activation of ouabain-sensitive Na,K-ATPase (data not shown). Because Glu stimulates free radical formation, one would also predict that manipulation of free radicals should also affect Na,K-ATPase. Consistent with this, xanthine/xanthine oxidase increased and SOD decreased activity. These effects appeared distinct from those of the Glu/CO pathway, although still involving an action on GC. Such effects may have relevance to the pathophysiology of cerebral ischemia.

#### **Complex Relationship of CO Metabolism to GC**

Although changes in Na,K-ATPase activity were associated with alterations in cGMP levels in vitro, the relationships between cGMP, Na,K-ATPase, and the CO system are likely to be much more complex in vivo. For example, in addition to CO itself, precursors to CO synthesis can also alter GC activity, as can heme, an HO-2 substrate formed from insertion of iron into protoporphyrin IX (Ignarro et al., 1982). Because concentrations of these intermediates in vivo is unknown, it is unclear whether such effects occur physiologically. Nonetheless, porphyrin pathway intermediates and their synthetic enzymes constitute possible additional sites for regulation of cGMP levels (and by implication, Na,K-ATPase activity) by the porphyrin/CO system.

It should also be noted that the direction of Na,K-ATPase modulation by cGMP may not be the same for all isoforms of Na,K-ATPase. For example, we have observed that in rat kidney, which contains the  $\alpha 1$  isoform of Na,K-ATPase, cGMP and hormones (such as acetylcholine) thought to act through NO formation cause down-regulation of Na,K-ATPase (McKee et al., 1994). An inhibitory response in kidney is also seen for atrial natriuretic factor, which activates membrane-associated GC (Scavone et al., 1995). Whether this is also true of the  $\alpha 1$  isoform in brain is not known and will require additional study. Because the distribution of various isoforms differs substantially among various tissues and species (Sweadner, 1993), it is possible that the direction of cGMP- or hormone-mediated modulation in various brain regions may not be the same in all animals. For example, bovine cerebellum shows a de-

crease in both cGMP and Na,K-ATPase in response to either CO or ACPD (Nathanson et al., unpublished data).

#### **Physiological Implications**

The biochemical linkage between CO, GC, and Na,K-ATPase, together with the colocalization of  $\alpha 3$ , cGMP, HO-2, and PKG in PNs, suggests that the PN is a possible site for intracellular regulation of Na,K-ATPase by CO, the initial steps of which involve HO-2-mediated formation of CO, stimulation of GC, and activation of PKG. The present results also suggest that Glu, acting at mGluRs, may regulate this system, possibly through an action on PKC and stimulation of HO-2 synthesis. The observed enrichment of  $\alpha 3$  Na,K-ATPase in the region of the PN axon hillock and recurrent PN axons has physiological implications, as it constitutes a localization where alteration of Na<sup>+</sup> pump activity, with consequent modulation of local membrane potential, could modify PN excitability and have significant effects on axonal firing (presumably hyperpolarizing and inhibitory if Na,K-ATPase is stimulated).

CO, however, may also diffuse out of cells and act intercellularly, either pre- or postsynaptically. Thus, PN Na,K-ATPase may also be subject to regulation by extracellular CO generated from HO-2, known to be present in granule and possibly other cell types (Verma et al., 1993). Conversely, CO produced in PNs or granule cells could affect other neurons or presynaptic terminals, as suggested, e.g., by our observation following CO exposure, of intense punctate cGMP immunofluorescence in basket cell synaptic endings terminating on PN cell bodies (see Figure 4F). Although it is unknown whether PKG is present in basket cell terminals, it is of interest that  $\alpha 3$  mRNA has been reported to be enriched in basket cell bodies (Hieber et al., 1991), and high affinity ouabain binding suggestive of basket cell terminals has been seen on PN cell bodies (Brines et al., 1990). The intense cGMP signal in these nerve endings could have functional implications, given recent evidence (Zhuo et al., 1994) in culture demonstrating that 8-Br-cGMP can increase quantal release of neurotransmitter. Enhanced release (of  $\gamma$ -aminobutyric acid) from basket cell terminals would have the effect of increasing inhibition of PNs.

In addition to a possible role in synaptic communication, released CO might also function as a local metabolic messenger responding to the general level of neural activity and regulating Na<sup>+</sup> pumps of neighboring neurons, as well as ion-buffering and neurotransmitter-sequestering glia. Maintenance of electrical and ionic gradients has implications for cell viability, particularly during periods of metabolic or excitotoxic stress (Beal et al., 1993; Coyle and Puttfarcken, 1993). Consistent with this possibility, inhibition of  $\alpha 2$  and  $\alpha 3$  Na,K-ATPase isoforms by brief exposure to low dose ouabain markedly potentiates Glu excitotoxicity in neuronal/glia cultures (Brines and Robbins, 1992). Furthermore, in hippocampal slices, addition of ouabain or reduction of ATP decreases uptake and can even cause release (via cotransporter reversal) of excitatory amino acids (Madl and Burgesser, 1993).

Whereas an increase in Na<sup>+</sup> pump activity might generally be beneficial, inappropriate or excessive stimulation

of Na,K-ATPase might itself contribute to cell stress, given the large energy requirements of ion pumping (Ames, 1991). It has been observed, e.g., that prolonged application of GluR agonists causes a marked depletion of ATP (Beal et al., 1993). The resulting AMP supplies purines that act as a substrate for xanthine oxidase, resulting in increased free radical formation. Xanthine/xanthine oxidase and free radicals can further activate Na,K-ATPase, thereby depleting ATP more and creating a vicious cycle. Accordingly, factors that modulate CO synthesis *in vivo* are not only of physiological interest but have pathophysiological implications related to the vulnerability of tissues to excitotoxic lesions.

Whether CO and cGMP (and possibly free radicals) acting through Na,K-ATPase might influence long-lasting synaptic effects related to learning or memory remains to be determined; however, of possible relevance are recent electrophysiological data, in hippocampus, showing that cGMP and PKG contribute to LTP, and that SOD, along with inhibitors of HO-2 and PKC (all agents that inhibited Na,K-ATPase activation in the present studies), can reverse or block LTP (Zhuo et al., 1994; Stevens and Wang, 1993). Similar data have also been described for LTD in cerebellum (e.g., Linden et al., 1993; Crepel and Jaillard, 1990). Also of relevance is the known presence of HO-2 in CA1 pyramidal cells and our additional observations that CO can elevate cGMP and stimulate Na,K-ATPase activity in hippocampal slices (Nathanson et al., unpublished data). Given the extensive involvement of ATP-dependent Na<sup>+</sup> pumps in ion transport, transmitter reuptake, cellular excitability, and cell energy metabolism, the present results support a potentially significant role for CO and, particularly, the modulation of Na,K-ATPase as a common focal point for physiological regulation in the nervous system.

## Experimental Procedures

### Na,K-ATPase

Effects of CO and drugs on Na,K-ATPase activity were measured in  $0.4 \times 0.4 \times 1$  mm slices of adult rat cerebellum prepared on a Brinkmann tissue chopper, washed extensively to remove small particles, cooled to 4°C, and suspended (25–30 mg/ml) in buffer containing 137 mmol/l NaCl, 5 mmol/l KCl, 0.8 mmol/l MgSO<sub>4</sub>, 0.25 mmol/l CaCl<sub>2</sub>, 1 mmol/l MgCl<sub>2</sub>, 10 mmol/l HEPES (NaOH [~2mM]) to adjust pH to 7.4 at 34°C. Following preincubation for 60 min at 34°C, high purity CO, argon, and/or drugs were added to tubes (five replicates per condition) containing 1 ml aliquots of slice suspension, incubated (15 min at 34°C), and then rapidly frozen on dry ice. Tissue was then measured for cGMP and Na,K-ATPase as previously described (McKee et al., 1994). Alteration of activity due to CO was maintained for at least 60 min. CO itself was added in a closed system in the form of an aqueous solution. To compensate for diffusional loss of gas during incubation, a second addition of CO or argon was made 7 min after the 15 min drug incubation started. In all experiments, CO and drugs were removed prior to ATPase assay.

### GC

GC activity was measured both in the pellet and high speed (100,000 g  $\times$  60 min) supernatant of rat cerebellum homogenized (200 mg/ml) in cold 10 mM Tris HCL (pH 7.4), containing 1 mM EDTA and 1 mM dithiothreitol. Tubes (300  $\mu$ l) contained 4 mM MgCl<sub>2</sub>, 80 mM Tris-maleate (pH 7.4), 10 mM theophylline, and 30  $\mu$ l of tissue fraction. The reaction (10 min at 30°C) was started with GTP (1 mM) and terminated by addition of 75 mM NaAc and heating to 90°C (3 min). cGMP

formed during the reaction was measured by RIA with appropriate drug blanks.

### Immunocytochemistry

Immunostaining, using rabbit polyclonal for cGMP (1:200), isoform specific Na,K-ATPase monoclonals (1:5; McGrail and Sweadner, 1991), and a monoclonal for the  $\gamma$  isoform (C-terminal) of PKC (Transduction Laboratories) was as previously described (Snyder et al., 1992), except that for cGMP, 5 mM IBMX was added to some slices, and primary antibody incubations varied from 1 to 18 hr. The rabbit polyclonal cGMP antibody (Fitzgerald, Concord, MA) had been generated to cGMP coupled to keyhole limpet hemocyanin and had less than 0.02% (1:5000) cross-over with cAMP and less than 0.000002% (1:50,000,000) cross-over with ATP. The antibody was tested and was found to label appropriately several tissues known to be enriched in cGMP. Control sections utilizing nonimmune serum in lieu of hybridoma culture supernatant yielded low backgrounds clearly distinct from the staining patterns noted for the specific antibodies. (The only exception was the rabbit polyclonal for cGMP, which showed some nonspecific staining of stellate and basket cell nuclei.) Diaphorase was run as previously described (McKee et al., 1994). See Figure 2 legend for quantitation of fluorescence.

### Capture Assay for Measurement of CO Production

The rate of CO production by HO was assayed in slices by using a spectrophotometric method which measured the rate of Hb-CO formation from oxyhemoglobin (J. A. N. and M. M., unpublished data). A key advantage of this assay was its direct measurement of CO production, rather than the use of an indirect measure of HO activity, such as the secondary conversion of biliverdin to bilirubin seen in some prior methods (Maines, 1988). Because compartmentalized oxyhemoglobin in the form of RBCs was used, the assay could measure HO activity both in slices and in soluble cell fractions without interference from porphyrin substrates or HO product. Conversely, the Hb capture agent did not interfere with HO enzymatic activity. During incubation, CO produced by HO diffused from the incubation medium or slice into the surrounding dilute suspension of RBCs, which captured CO in the form of Hb-CO. Slices were separated by settling, and RBCs were isolated by centrifugation and washing. Hb-CO in lysed RBCs was then measured spectrophotometrically using a four wave length algorithm and an automated modified model IL482 CO-Oximeter. More detailed spectrophotometric analysis revealed the expected spectral shift of Hb-CO from oxyhemoglobin at approximately 578 nm. Using standards prepared from saturated gas solutions of known composition, cross-over from Hb-NO was experimentally determined to be <4% than that of CO. Because endogenous tissue production of NO is usually far less than that of CO, interference from Hb-NO was insignificant. In the present study, slices prepared as described above for Na,K-ATPase were incubated for 20 min at 34°C at a total effective Hb concentration of 0.16 g per 100 ml.

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